Immunomodulator control of Myofibroblast Plasticity & Nuclei Morphology

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INTRODUCTION

Significance and Background

- Scarring is associated with extra-cellular matrix (ECM) dysregulation[1] and myofibroblast activation and persistence.[2]
- Myofibroblasts are a contractile pro-fibrotic cell type critical for early remodeling and deposition of predominantly type I collagen after injury in adult healing soft tissues.[1,2,3]
- Treatment strategies that decrease myofibroblasts are critical for regulating matrix remodeling for regeneration over scar-mediated repair.[9,10,11]
- Although the mechanisms are not fully understood, targeting the NF-κB signaling pathway has potential to disrupt myofibroblast pro-survival signaling which contributes to fibrotic disease.[4]
- The small molecule IKKβ inhibitor, 2-Amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-(4-piperidinyl)-3 pyridinecarbonitrile (ACHP) blocks IKKβ, which shuts down the inflammatory arm of the NF-κB signaling pathway.[6]
- Recently, NF-κB inhibition with via ACHP has been shown to reduce myofibroblast activation and inflammatory signaling *in vitro*[8] and promote tendon healing *in vivo*.[6]

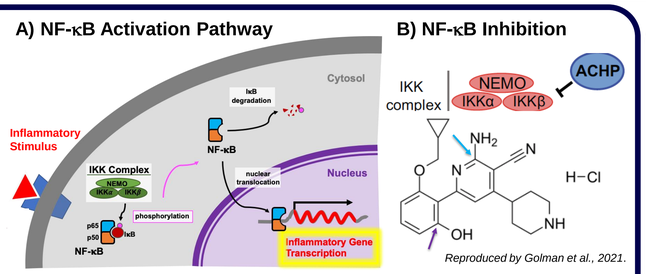

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Figure 1: *A) NF-κB in myofibroblasts of human tendon scar.* ***A) The Canonical NF-κB Signaling Pathway.*** *Inflammatory stimuli signal through receptor specific interactions to recruit IκB Kinase (IKK) complex which phosphorylates IκB marking it for degradation. Subsequently, unbound NF-κB dimers are free to undergo nuclear translocation to activate transcription of inflammatory genes (adapted from Murphy & Weaver, 2016 ; Abraham et al.).* ***B)*** *ACHP inhibition of IKKβ and ACHP chemical structure.*

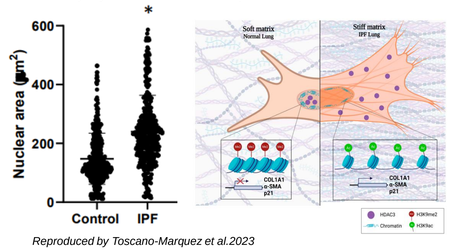

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Figure 2*: Matrix stiffness causing chromatin remodeling in IPF lung cells, that contributes to enlargement of nuclei size.*

Objectives and Hypothesis

- The objective was to determine the *in vitro* anti-fibrotic potential of IKKβ inhibitor, ACHP Hydrochloride and minimum effective dosage to control myofibroblast plasticity *in vitro.*
- In addition, recent findings of alterations in chromatin compaction dynamics with fibrosis implicates enlarged nuclei size as a marker for fibrotic disease, thus this study also aims to explore nuclei morphology associated with myofibroblast activation and ACHP treatment.[8]
- It was hypothesized that all three doses will de-sensitize fibroblasts to TGF-β1 induced myofibroblast differentiation (indicated by decreased α-SMA expression), and NF-κB activation; while nuclei size will correlate with myofibroblast presence and therefore decrease in dose-dependent manner with ACHP treatment.

MATERIALS & METHODS

- *In vitro* Experiment. Human hTERT lung fibroblasts were expanded *in vitro* culture until passage 5, seeded (20,000 cells/cm²) and stimulated after 24 hours of adherence with fibrotic cytokine TGF-β1 (10 ng/mL) for either 48 hours (Group G1) or throughout the remainder of the experiment (Group T1). A known initiator of NF-κB signaling, ACHP hydrochloride was used to exogenously treat cells simultaneously at three different dosages [1,10,100 μM].
- Cellular Behavior. Cell number and proliferation (n=5/group/timepoint) was assessed using the LIVE/DEAD™ Viability/Cytotoxicity assay (Live = Green; Dead = Red) and Quant-iT™ PicoGreen™ dsDNA assay
- Cellular Morphology & Phenotype. Myofibroblast presence and NF-κB activation were assessed through α-smooth muscle actin (α-SMA) and phospho-p65 immunofluorescence, respectively. All samples were imaged at 40x magnification via Olympus Confocal Imaging. ImageJ analysis was performed to quantify the percent of differentiated myofibroblasts as well as cytosolic versus nuclear localization of phospho-p65 fluorescence (n=3 replicates/group; n>5 images/replicate quantified).
- Characterization of Nuclei Morphology. DAPI (4′,6-diamidino-2-phenylindole) staining was utilized to quantify cell number and nuclei morphology by area and perimeter in ImageJ.

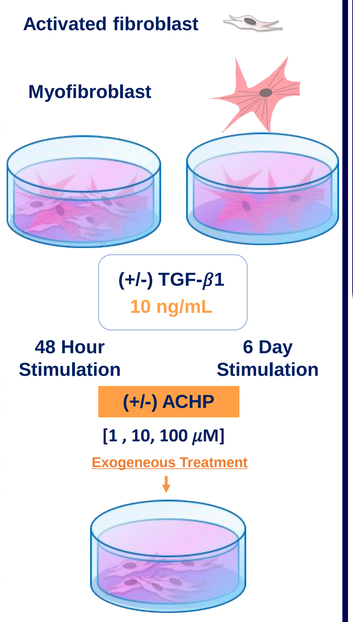

Figure 3*: Study Design schematic and expected outcomes.*

RESULTS

- Cell Viability & Proliferation: Cell number decreased in dose-dependent manner with [ACHP] dose. The highest [100 μM] ACHP dose was cytotoxic. All cells proliferated overtime.

A. Cell Viability & Proliferation with exogenous ACHP treatment

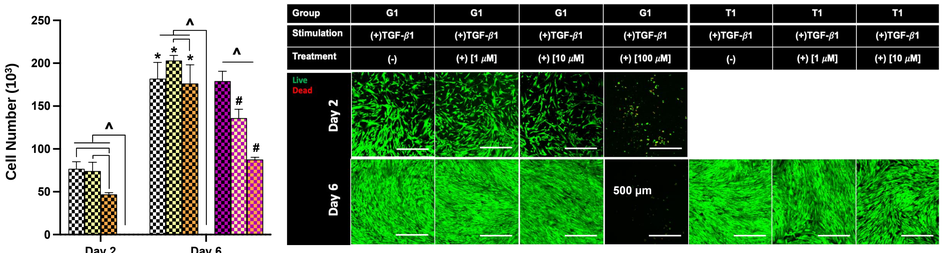

Figure 4. *Cell viability and proliferation over 6 days in vitro.*

B. Myofibroblast & NF-κB Activation

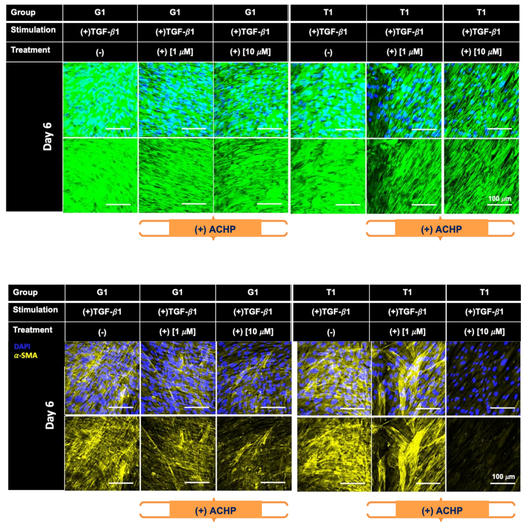

Figure 5 *Myofibroblast and NF- κB over 6 days in vitro.*

- Myofibroblast & NF-κB Activation: Continued TGF-β1 stimulation increased α-SMA stress fiber formation and NF-κB activation, while ACHP treatment reduced both α-SMA expressing myofibroblast presence and NF-κB activation in a dose-dependent manner.

C Quantification of immunofluorescence with ImageJ

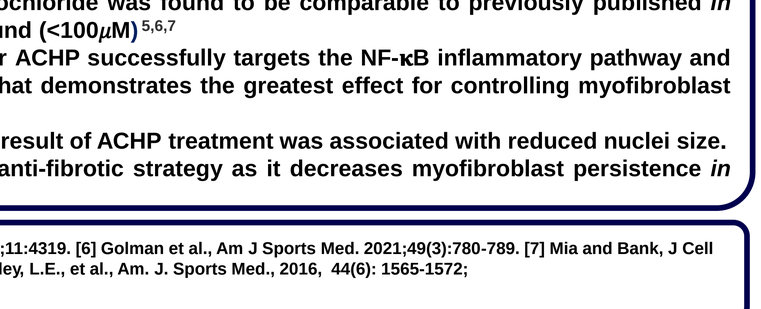

Figure 6. *Day 6 Quantification of Immunofluorescence*

- Characterization of Nuclei Morphology: Nuclear size increases with continued TGF-β1 stimulation as expected and decreases with ACHP treatment in a dose-dependent manner by Day 6.

D Quantification of Nuclei Morphology with ImageJ

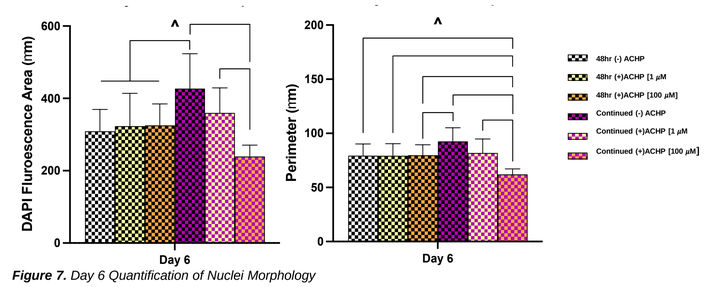

Figure 7. *Day 6 Quantification of Nuclei Morphology*

DISCUSSION & CONCLUSIONS

- Nuclei size, activated NF-κB and α-SMA stress fiber formation increased with duration of TGF-β1 stimulation as expected.
- An acceptable target dosage of ACHP hydrochloride was found to be comparable to previously published *in vitro* culture with the small molecule compound (<100μM)[5,6,7]
- We demonstrated that the immunomodulator ACHP successfully targets the NF-κB inflammatory pathway and that 10 μM is the maximum tolerated dose that demonstrates the greatest effect for controlling myofibroblast plasticity.
- In addition, reduction in myofibroblasts as a result of ACHP treatment was associated with reduced nuclei size.
- Overall, ACHP Treatment offers a potential anti-fibrotic strategy as it decreases myofibroblast persistence *in vitro.*

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